Protein–S–S–Glutathione Mixed Disulfides as Models of Unfolded Proteins†

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ABSTRACT: Mixed disulfides between glutathione and the reduced forms of disulfide-bonded proteins were generated and characterized to explore their suitability as models of the unfolded state of newly-synthesized secretory proteins. RNase T_1 and α-lactalbumin were reduced and converted to mixed disulfide derivatives, named GS–RNase T_1 and GS–α-lactalbumin, in good yield; the molecular masses of the derivatives were confirmed by electrospray mass spectrometry. The intrinsic fluorescence of the derivatives and the binding of the hydrophobic fluorescent dye ANS were characteristic of fully unfolded proteins. Fluorescence studies and enzyme activity data indicated that GS–RNase T_1 could be refolded to a nativelike state at NaCl concentrations greater than 1.5 M, as was previously demonstrated for the reduced, carboxymethylated derivative of this protein. The [NaCl]-dependent folding/unfolding equilibrium for GS–RNase T_1 was reversible and could be influenced by urea. Fluorescence studies indicated that GS–α-lactalbumin showed a [NaCl]-dependent partial shift toward a more nativelike state, which was enhanced by the presence of Ca^{2+} ions. Both of the GS derivatives stimulated the ATPase activity of BiP, with apparent affinities in the range 0.1–1.0 mM. The results indicate that these GS–S–protein mixed disulfide derivatives are ideal model unfolded proteins that can be used as substrates for detailed studies on secretory protein folding *in vitro* and on the interactions between unfolded proteins and facilitators of protein folding.

Almost all secretory proteins contain disulfide bonds that are important for the stability and/or function of the folded proteins (Freedman, 1984). The folding of secretory proteins is therefore accompanied by disulfide bond formation, which takes place in the endoplasmic reticulum (ER[1]) (Creighton et al., 1993); the ER lumen in fact is unique among folding compartments in the eukaryotic cell, since it provides an oxidizing environment (Hwang et al., 1992). The formation of protein disulfide bonds within the ER is essential for subsequent intracellular transport and maturation (Braakman et al., 1992). Our aim is to develop a model of the initial folding of S–S-containing proteins at biosynthesis that is realistic in chemical and cellular terms, taking into account the properties of major resident proteins in the ER lumen, such as BiP and PDI (Rowling & Freedman, 1993). A definition of the mechanism of action of these two proteins and any possible synergistic activity between them will be crucial for understanding the mechanism of protein folding within the ER.

Reduced and carboxymethylated proteins have been used as models of unfolded proteins that remain unfolded in "native" conditions (Langer et al., 1992). In order to study the refolding of secretory proteins, analogous unfolded species are required that are stable but can nevertheless refold and form native disulfides in controlled conditions. We have explored mixed disulfides between reduced unfolded proteins and glutathione as models of unfolded secretory proteins. Naturally-occurring mixed disulfides have been found between proteins and glutathione (Huisman et al., 1962); some mutant vertebrate lysozymes are secreted from yeast as glutathione mixed disulfides that are susceptible to thiol/disulfide interchange (Hayano et al., 1993). Glutathione is in fact the most abundant non-protein thiol in mammalian cells and has been identified as the principal redox buffer in the ER lumen (Hwang et al., 1992), although how the redox state of this compartment is maintained is still unknown (Freedman, 1990).

Two proteins, RNase T_1 and α-lactalbumin, were selected to generate the mixed disulfides; both proteins are suitable subjects for studying the folding process and protein structure in general. RNase T_1 is a small protein of 104 amino acids containing two disulfide bonds, Cys2–10 and Cys6–103; α-lactalbumin instead has 123 amino acids and four disulfide bonds, Cys6–120, Cys28–111, Cys61–77, and Cys73–91. The mixed disulfides, named GS–RNase T_1 and GS–α-lactalbumin, were generated, characterized chemically by mass spectrometry, showing that each cysteine residue had reacted with exogenous glutathione, and found to be unfolded in native conditions by intrinsic and extrinsic fluorescence analysis.

We then sought to analyze the conformational behavior of the mixed disulfide derivatives by seeking conditions in which to refold them without native disulfide formation, i.e., while preventing thiol/disulfide interchange by the exclusion of thiol compounds. The folding behavior of GS–RNase T_1 and GS–α-lactalbumin was therefore monitored by intrinsic fluorescence in the presence of NaCl; the analysis on GS–RNase T_1 was carried out in parallel with that on the reduced and carboxymethylated form, as it has been shown to refold in the presence of high concentrations of NaCl (Pace et al., 1988a). The folding behavior of GS–α-lactalbumin in the presence of NaCl was analyzed by intrinsic fluorescence under conditions

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[1] Abbreviations: ANS, 1-anilinonaphthalene-8-sulfonate; ATP, adenosine 5′-triphosphate; BiP, immunoglobulin heavy chain binding protein; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; EDTA, ethylenediamine tetraacetic acid, disodium salt; ER, endoplasmic reticulum; ES/MS, electrospray mass spectrometry; GSSG, oxidized glutathione; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high-performance liquid chromatography; IAA, iodoacetic acid; LDH, lactate dehydrogenase; λ_{max}, wavelength of maximum fluorescence emission; NAD^+, nicotinamide adenine dinucleotide (oxidized); NADH, nicotinamide adenine dinucleotide (reduced); PDI, protein disulfide isomerase; PEP, phosphoenolpyruvate; PK, pyruvate kinase; TFA, trifluoroacetic acid; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol.

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that stabilize or destabilize the native state by exploiting the fact that the native protein contains a single tightly-bound Ca^{2+} ion that specifically stabilizes the native conformation. This effect of Ca^{2+} therefore makes it possible to stabilize or destabilize the native conformation by varying the free Ca^{2+} concentration (Mitani et al., 1986).

The interaction of the protein mixed disulfides with BiP, the ER luminal member of the hsp70 family, was finally studied as a model of its interaction with unfolded proteins; the analysis was carried out in parallel with that on the native proteins. BiP, as well as the other members of the hsp70 family, is in fact able to distinguish between folded and unfolded polypeptides, forming no interactions with the native form of a protein (Palleros et al., 1991).

MATERIALS AND METHODS

RNase T_1 was a generous gift of N. Pace (Texas A&M University Health Science Center). ANS (magnesium salt), bovine milk α-lactalbumin, DTNB, GSSG, guanidinium chloride, Hepes, IAA, LDH, NADH, NAD^+, PEP, PK, and yeast RNA were obtained from Sigma Chemical Co.; ATP, EDTA, and Tris were purchased from Boehringer Mannheim GmbH; the FPLC prepacked Sephadex G-25 column was acquired from Pharmacia. All other reagents were HPLC grade from Fisons.

Preparation of the Mixed Disulfide Derivatives (GS–RNase T_1 and GS–α-Lactalbumin). RNase T_1 and α-lactalbumin were reduced at a concentration of 10 mg/mL in 0.1 M Tris-HCl and 1 mM EDTA (pH 8.5) containing 6 M guanidinium chloride by incubation with reduced DTT for 2 h at 37 °C under a nitrogen atmosphere (DTT mol/S–S mol = 50/1). After the addition of 0.2 vol of 1 M HCl, the proteins were separated from excess reagent by rapid gel filtration on a Sephadex G-25 column, equilibrated, and eluted with 0.01 M HCl. The protein fraction was then recovered, tested for SH content, and lyophilized.

The fully reduced proteins (2 mg/mL) were treated with GSSG (neutralized with Tris-base) in 0.5 M Tris-HCl and 1 mM EDTA (pH 8.5) containing 6 M guanidinium chloride at room temperature for 3–10 h, under a nitrogen atmosphere (GSSG mol/protein mol = 1000/1). The reaction mixture was acidified with 0.2 vol of 1 M HCl and desalted on a Sephadex G-25 column eluted with 0.01 M HCl. The protein fraction was recovered, tested for SH content, and lyophilized. The materials were stored at −20 °C.

The concentration of solutions of native RNase T_1 and all of the modified forms described was determined using an absorption of 1.67 at 278 nm for 1 mg/mL solution (Pace et al., 1988a). The concentration of solutions of native and modified forms of α-lactalbumin was determined using an extinction coefficient at 280 nm of 28 500 M^{-1} cm^{-1} (Kuwajima et al., 1990).

Preparation of Reduced and Carboxymethylated RNase T_1 (CM-RNase T_1). RNase T_1 was reduced at a concentration of about 1 mM in 0.1 M Tris-HCl and 1 mM EDTA (pH 8.5) containing 6 M guanidinium chloride by incubation with reduced DTT (DTT mol/S–S mol = 100/1) for 3 h at 37 °C, under a nitrogen atmosphere. After the addition of 0.2 vol of 1 M HCl, the reaction mixture was chromatographed on a Sephadex G-25 column equilibrated and eluted with 50 mM ammonium bicarbonate (pH 8.0) containing 10 mM DTT. The protein fraction was recovered and lyophilized.

Reduced RNase T_1 was carboxymethylated with a 0.7 M iodoacetic acid (IAA) solution in 0.3 M Tris-HCl and 1 mM EDTA (pH 8.0) containing 6 M guanidinium chloride for 30 min, at room temperature, in the dark (IAA mol/total SH mol = 10/1). The protein was then separated from the excess blocking reagent on a Sephadex G-25 column equilibrated and eluted with 50 mM ammonium bicarbonate (pH 8.0). The protein fraction was recovered, tested for SH content, and lyophilized. The material was stored at −20 °C.

SH Titration. The free SH content of all of the modified forms of RNase T_1 and α-lactalbumin was estimated by DTNB titration in 0.3 M Tris-HCl and 1 mM EDTA (pH 7.5) containing 6 M guanidinium chloride, and the formation of the 2-nitro-5-thiobenzoate dianion was measured at 412 nm (extinction coefficient, 13 600 M^{-1} cm^{-1}) (Ruoppolo et al., 1993).

HPLC Analysis. The native and modified proteins were analyzed by HPLC using a Vydac 218TP54 reversed-phase C_{18} column (0.46 × 25 cm^2); the elution system consisted of 0.1% TFA in water (solvent A) and 0.07% TFA in acetonitrile (solvent B). Native and modified proteins were eluted with a linear gradient of solvent B from 5% to 65% at a flow rate of 1 mL/min. Protein monitoring was carried out at 220 nm.

Electrospray/Mass Analysis. The native and derivatized proteins were collected from analytical HPLC runs and directly submitted to ES/MS analysis using a VG BIO Q triple quadrupole mass spectrometer (C.E.I.N.G.E, Protein Chemistry Unit, c/o Servizio di Spettrometria di Massa del CNR, Università di Napoli, Napoli, Italy). Protein samples (10 μL), at a concentration of 10 pm/μL, were injected into a fused silica capillary tube through a Rheodyne injection valve and introduced into the ion source at a flow rate of 2 μL/min. The spectra were scanned at 10 s/scan from 1200 to 2200 m/z for the native RNase T_1, from 800 to 1800 m/z for the GS–RNase T_1 and the CM-RNase T_1, from 1100 to 1800 m/z for the native α-lactalbumin, and finally from 700 to 1400 m/z for the GS–α-lactalbumin. Mass-scale calibration was performed by means of the multiply charged ions from a separate injection of myoglobin or lysozyme (average molecular masses, 16 950.6 and 14 305.99 Da, respectively).

Fluorescence Analysis. The emission spectra were recorded between 280 and 450 nm, with bandwidths of 5 nm, in 1-cm-square cuvettes in a Perkin-Elmer LS-5B spectrofluorimeter at 25 °C. The intrinsic fluorescence of 0.54 μM solutions of RNase T_1, GS–RNase T_1, and CM-RNase T_1 was recorded after excitation at 278 or 295 nm (10-nm bandwidth); the emission spectra of 1.8 μM solutions of α-lactalbumin and GS–α-lactalbumin were recorded after excitation at 280 or 295 nm (10-nm bandwidth). Only fresh made protein solutions were used.

The protein solutions were incubated in 20 mM Hepes (pH 7.0) in the presence of various concentrations of NaCl at 25 °C for 6 or more hours to ensure that equilibrium was reached before the measurements were made. α-Lactalbumin and GS–α-lactalbumin were, in addition, incubated in 20 mM Hepes (pH 7.0) containing 10 mM $CaCl_2$ or 1 mM EDTA in the presence of various concentrations of NaCl at 25 °C for 6 h before the spectra were recorded.

In another set of experiments, RNase T_1, GS–RNase T_1, and CM-RNase T_1 were incubated in 20 mM Hepes (pH 7.0) containing 1.5, 2.0, 2.5, and 3.0 M NaCl for 6 h and then dialyzed against 20 mM Hepes (pH 7.0) overnight. The fluorescence measurements were made on the dialyzed solutions.

Urea stock solutions were prepared, and their concentrations were determined as described (Pace, 1986); only freshly made urea-containing solutions were used. RNase T_1, GS–RNase T_1, and CM-RNase T_1 were incubated in 20 mM Hepes (pH

7.0) containing 0 or 1.5 M NaCl in the presence of either 1 or 3 M urea at 25 °C for at least 6 h before the spectra were recorded.

All of the intrinsic fluorescence readings were corrected for the fluorescence of the buffer solutions.

ANS Binding Analysis. RNase T_1 and GS–RNase T_1 were dissolved in 20 mM Hepes (pH 7.0) and 100 μM ANS; α-lactalbumin and GS–α-lactalbumin were dissolved in 20 mM Hepes (pH 7.0) and 50 μM ANS containing 10 mM $CaCl_2$ or 1 mM EDTA. The concentration of the ANS solution was determined using an extinction coefficient at 350 nm of 5000 M^{-1} cm^{-1} (Weber & Young, 1964). The protein concentration was varied by dilution in the same buffer, keeping the ANS concentration constant and avoiding inner filter effects (Mulqueen & Kronman, 1982). The emission spectra were recorded between 400 and 650 nm (10-nm bandwidth) after excitation at 350 nm (10-nm bandwidth) at 25 °C. The fluorescence emission at 490 nm was measured after correction for background fluorescence caused by ANS in the absence of proteins.

RNase T_1 Activity Assay. Native and modified RNase T_1 activity was measured by recording the decrease in A_{260} at 30 °C of an 82 μg/mL RNA solution in 50 mM Tris-HCl, 25 mM KCl, and 5 mM $MgCl_2$ (pH 7.5) containing 0 or 1.5 M NaCl. Only freshly made RNA solutions were used.

Samples were incubated in 20 mM Hepes (pH 7.0) containing either 0 or 1.5 M NaCl at 25 °C for at least 6 h before the activity assays were made. RNA solution (3 mL) was preincubated in the spectrophotometer for 2 min and checked for the absence of autohydrolysis. Then the protein solutions were added to start the reaction. The final concentration of RNase T_1 was 10 nM and those of GS–RNase T_1 and CM-RNase T_1 were 0.16 μM.

The decrease in absorbance was measured for 5 min, and the value of ΔA_{260}/min was used as a measure for the enzymatic activity.

BiP ATPase Activity Assay. BiP was purified from bovine liver microsomes by a procedure (Rowling et al., 1994) modified from that of Flynn et al. (1989). GS–RNase T_1 and GS–α-lactalbumin were dissolved in 20 mM Hepes (pH 7.0) and added (0–1 mM final concentration) to BiP (4 μg). The native underivatized proteins were used in the control assay.

BiP ATPase activity was determined by a coupled assay, using a combination of pyruvate kinase, lactate dehydrogenase, and NADH (Price et al., 1993). The mixture of lactate dehydrogenase and pyruvate kinase obtained from Sigma was diluted 2-fold with 20 mM Hepes (pH 7.0), and 2 μL was added for each assay. ATP, $MgCl_2$, NADH, and PEP were dissolved in 20 mM Hepes (pH 7.0) at the following concentrations: 14 mM ATP, 14 mM $MgCl_2$, 1 mM NADH, and 7 mM PEP. Then 11 μL of this solution was finally added to the reaction mixture (50 μL, final volume), and the reactions were followed spectrophotometrically in a Beckman DU-70 spectrophotometer thermostated at 30 °C, recording the decrease in A_{340}. A 0.5 mM NAD^+ solution was used as a blank. The basal BiP ATPase activity in the absence of protein mixed disulfides or other derivative was subtracted for each protein concentration.

The kinetics parameters were calculated with the help of the program Enzfitter (Elsevier Biosoft, Cambridge, U.K.).

RESULTS

Chemical Characterization of GS-RNase T_1, CM-RNase T_1, and GS-α-Lactalbumin. RNase T_1 and α-lactalbumin were reduced under denaturing conditions and reacted with a 1000-fold excess of GSSG to generate the mixed disulfides. The free SH group content of GS–RNase T_1 and GS–α-lactalbumin was estimated by DTNB titration under denaturing conditions, showing that no thiol groups (less than 0.2 SH group/molecule) were present in the modified proteins. The preparation was carried out with a final yield of about 60% (mg of mixed disulfide/mg of starting native protein) for both the proteins. RNase T_1 was, in addition, reduced under denaturing conditions and reacted with IAA to generate the CM-RNase T_1, which was shown to have no thiol groups (less than 0.3 SH group/molecule) by DTNB titration.

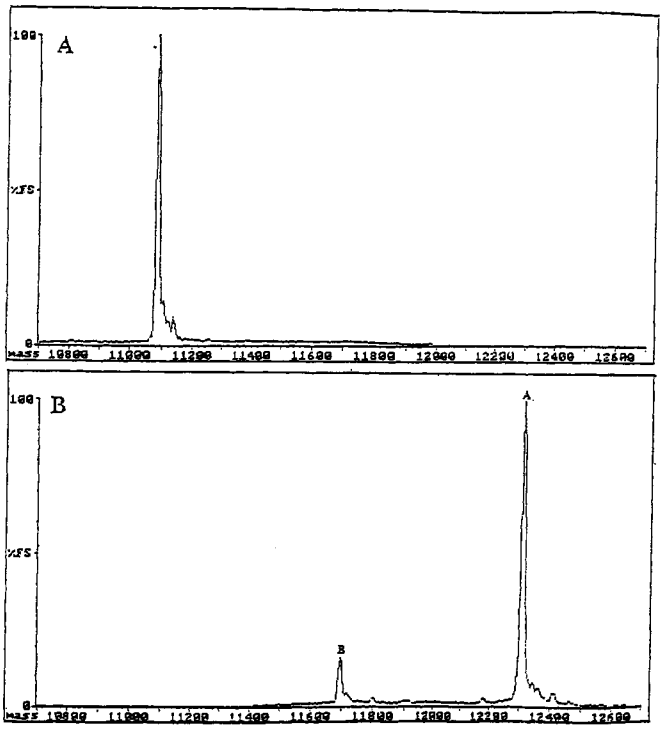

FIGURE 1: Electrospray mass spectrometric analysis of (A) native RNase T_1 and (B) GS–RNase T_1. The molecular mass measured for native RNase T_1 is 11 083.51 ± 1.11 Da. The measured molecular masses in the GS–RNase T_1 preparation are 12 307.15 ± 1.28 and 11 694.71 ± 3.82 Da, respectively.

Submitted to HPLC analysis on a reversed-phase C_{18} column, under the conditions described in the Materials and Methods section, native RNase T_1 exhibited a peak with a retention time of 33.6 min, whereas GS–RNase T_1 and CM-RNase T_1 were eluted at 32.8 and 34.2 min, respectively. Native α-lactalbumin was eluted after 39.1 min and GS–α-lactalbumin after 37.2 min. The modified proteins were collected from HPLC runs and directly submitted to electrospray/mass spectrometric analysis in order to provide a structural characterization of the molecular species obtained. The same analysis was also performed on the native proteins as a reference. The recorded mass spectra showed the characteristic bell-shaped distribution of multiply charged ions, from which the molecular mass of the proteins could easily be calculated. These multiply charged ion spectra can be transformed on a real mass scale, as shown in Figures 1 and 2.

The molecular weight of native RNase T_1 (Figure 1A) was measured as 11 083.51 ± 1.11 Da, which agrees with the predicted value (11 084.66 Da). Figure 1B shows the electrospray mass spectrum of the GS–RNase T_1 transformed on a real mass scale. A major component (component A, 90%) was detected with a molecular mass measured as 12 307.15 ± 1.28 Da. This mass value agrees with that

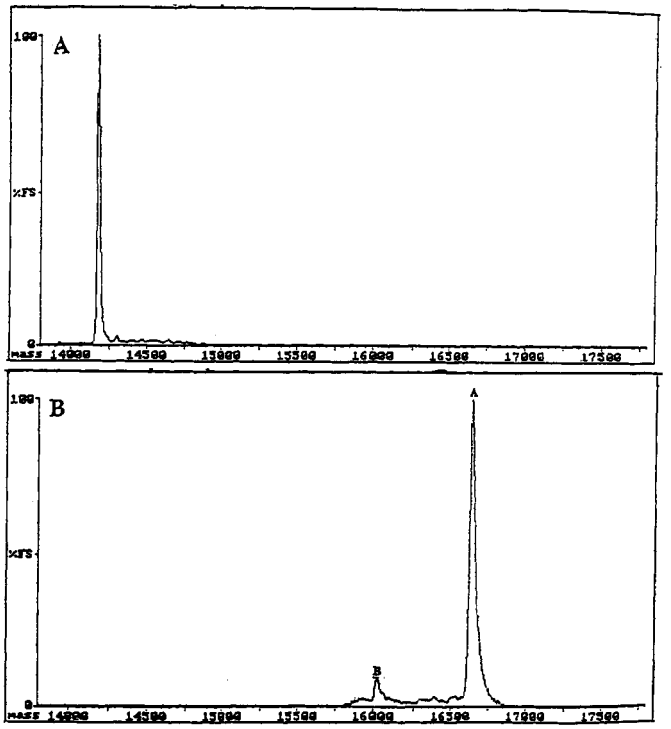

FIGURE 2: Electrospray mass spectrometric analysis of (A) native α-lactalbumin and (B) GS–α-lactalbumin. The native protein shows a molecular mass of 14 180.98 ± 1.29 Da. The molecular masses measured in the GS–α-lactalbumin preparation are 16 635.08 ± 0.8 and 16 023.16 ± 3.79 Da, respectively.

predicted for the GS–RNase T_1 (12309.91 Da), thus demonstrating that all four cysteine residues present in the RNase T_1 had been converted to mixed disulfides with exogenous glutathione under the experimental conditions described. The ES/MS spectrum in fact showed the presence of only a single other minor component (component B, 10%) exhibiting a molecular mass of 11 694.71 ± 3.82 Da, which is 611.22 mass units higher than that obtained for the native protein. On the basis of the molecular mass of glutathione (307.32 Da) and the DTNB titration data (less than 0.2 thiol group/molecule present in the GS–RNase T_1 preparation), component B, which is only 10% of the whole preparation, was identified as a modified form of RNase T_1 in which two cysteine residues had been converted to mixed disulfides with exogenous glutathione, while the two remaining cysteines formed an intramolecular disulfide bridge. Finally, CM-RNase T_1 showed a molecular mass of 11 321.26 ± 1.01 Da, which is 237.75 mass units higher than that of the native protein, thus confirming that all four cysteines were blocked by the carboxymethyl group.

The ES/MS spectra of native α-lactalbumin and GS–α-lactalbumin, transformed on a real mass scale, are shown in Figure 2. The molecular mass obtained for the native protein (Figure 2A) was 14 180.98 ± 1.29 Da, which agrees with the predicted value (14 178.98 Da). The GS–α-lactalbumin preparation was greater than 90% pure, as judged by the ES/MS analysis. In fact, a major molecular species (component A, 94%) could be distinguished in the preparation (Figure 2B), showing a molecular mass of 16 635.08 ± 0.8 Da, which is 2454.1 mass units higher than that obtained for the native protein. These data clearly indicate that all eight cysteine residues present in the α-lactalbumin chain were derivatized with the exogenous glutathione molecules under the procedure described. Only a very minor component (component B, 6%) was detected, which exhibited a molecular weight of 16 023.16 ± 3.79 Da; this value is 1842.18 mass units higher than the

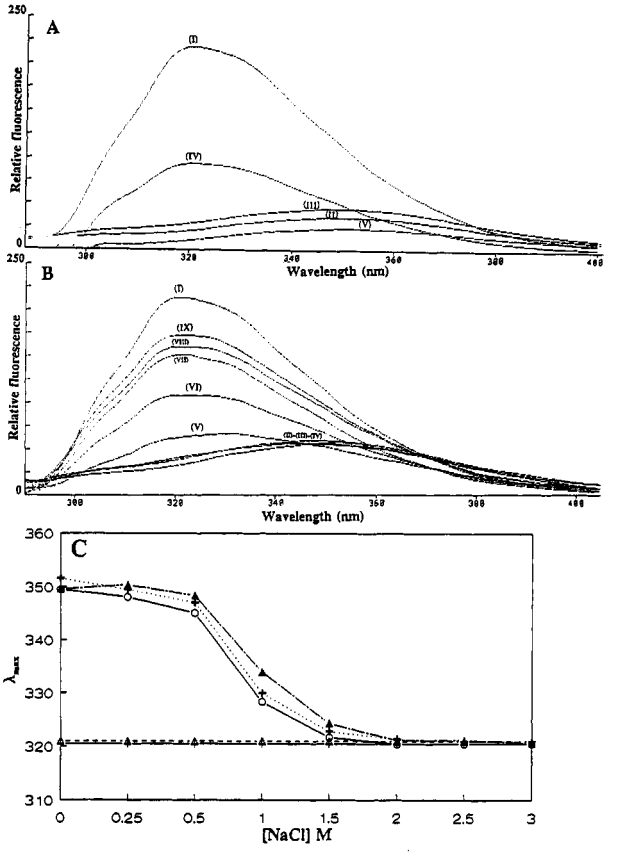

FIGURE 3: (A) Fluorescence emission spectra of native RNase T_1 (I and IV), GS–RNase T_1 (II and V), and CM-RNase T_1 (III) in 20 mM Hepes (pH 7.0). Fluorescence emission was measured after excitation at 278 (I, II, and III) or 295 nm (IV and V) at a protein concentration of 0.54 μM. (B) Fluorescence emission spectra of native RNase T_1 under standard conditions (I) and of GS–RNase T_1 in the presence of 0 (II), 0.25 (III), 0.5 (IV), 1.0 (V), 1.5 (VI), 2.0 (VII), 2.5 (VIII), and 3 M (IX) NaCl, after excitation at 278 nm. (C) λ_{max} values of RNase T_1 (+, excitation at 278 nm; △, excitation at 295 nm), GS–RNase T1 (○, excitation at 278 nm; +, excitation at 295 nm), and CM-RNase T1 (▲, excitation at 278 nm) are plotted versus varying concentrations of NaCl.

molecular mass of the native protein. The DTNB data (less than 0.2 SH group/molecule present in the GS–α-lactalbumin preparation) and the ES/MS analysis therefore indicate that 6% of the entire GS–α-lactalbumin preparation carries six cysteines derivatized with the exogenous glutathione and that the two remaining cysteines are involved in an intramolecular disulfide bridge. The mixed disulfide derivatives of both RNase T_1 and α-lactalbumin both comprised over 90% of a single fully derivatized species lacking free thiols or intramolecular disulfide bonds; they were used without further purification.

Fluorescence Analysis of RNase T_1, GS-RNase T_1, and CM-RNase T_1 in the Presence of NaCl. Figure 3A shows the fluorescence spectra obtained for the native and modified proteins under standard conditions (20 mM Hepes, pH 7.0), using excitation wavelengths of 278 and 295 nm. When excited at 278 nm, native RNase T_1 shows intense intrinsic fluorescence with a λ_{max} at 320.5 nm, whereas CM-RNase T_1 and GS–RNase T_1 show much less fluorescence intensity with λ_{max} values at 348.6 and 349.6 nm, respectively, corresponding to the λ_{max} of free tryptophan (348 nm). When excited at 295 nm, the λ_{max}'s of native RNase T_1 and GS–RNase T_1 showed slight red shifts to 321 and 351.5 nm, respectively, accompanied by a roughly 2-fold decrease in the fluorescence intensity. The similarity in emission spectra observed with excitation at 295 nm (excitation of Trp only) and at 278 nm (excitation of

Tyr and Trp) confirms that the major contribution to the intrinsic fluorescence emission of RNase T_1 is due to the single Trp (Trp59) buried in the interior of the molecule. The observed parameters, λ_{max} and relative fluorescence emission, demonstrate that GS–RNase T_1 and CM-RNase T_1 are in a denatured state, as the native protein was shown to have a much greater intrinsic fluorescence than the denatured form (Pace, 1986).

RNase T_1, GS–RNase T_1, and CM-RNase T_1 were incubated at 0.54 μM (final concentration) in 20 mM Hepes (pH 7.0) containing 0, 0.25, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 M NaCl at 25 °C for 6 or more hours, and then the intrinsic fluorescence emission spectra of the protein solutions were measured after excitation at 278 or 295 nm, 25 °C. Figure 3B shows the fluorescence spectra obtained for GS–RNase T_1 in the presence of varying concentrations of NaCl, after excitation at 278 nm. GS–RNase T_1 shows no significant changes in the λ_{max} values at 0.25 and 0.5 M NaCl, but the emission maximum is shifted from 346.1 to 329.6 as the concentration of NaCl is increased to 1.0 M. The modified protein regains, finally, the λ_{max} of the native form at 1.5 M NaCl. No further significant changes were detected on increasing the concentration of NaCl up to 3 M. In addition to the regain of the native λ_{max}, GS–RNase T_1 shows an increase in the intrinsic fluorescence as the NaCl concentration increases without complete recovery of the fine structure of the spectrum of the native form, which is probably due to the effect of the exogenous groups present in the molecule. Figure 3C shows the titration using excitation wavelengths of 278 and 295 nm; the graph clearly illustrates that GS–RNase T_1 acquires the native λ_{max} in the presence of high concentrations of NaCl. As expected, the data obtained with excitation at the alternative wavelengths are comparable; when excited at 295 nm, GS–RNase T_1 exhibited a minimal red shift in its λ_{max} at all concentrations of NaCl, together with a 2-fold decrease in the fluorescence intensity. When incubated in the presence of increasing concentrations of NaCl, the native protein shows no changes in the intrinsic fluorescence or in the λ_{max} due to the presence of the salt, whether excited at either 278 or 295 nm, as shown in Figure 3C.

As a control, the same kind of analysis was performed on the CM-RNase T_1, as CM-RNase T_1 has been shown to refold in the presence of high concentrations of NaCl (Pace et al., 1988). CM-RNase T_1 shows, in fact, the shift of the λ_{max} from 348.4 to 334 in the presence of 1 M NaCl and regains the native λ_{max} at 1.5 M NaCl, without any significant changes upon increasing the concentration of NaCl up to 3 M, as illustrated by the curve in Figure 3C. The CM-RNase T_1 also shows an increase in the intrinsic fluorescence as the concentration of NaCl increases, without completely recovering the native intrinsic fluorescence.

Finally, GS–RNase T_1 and CM-RNase T_1 were incubated in 20 mM Hepes (pH 7.0) containing 1.5, 2.0, 2.5, and 3.0 M NaCl for 6 h, and then the fluorescence spectra were recorded. Both GS–RNase T_1 and CM-RNase T_1 showed the regain of the native λ_{max} at those concentrations of NaCl, as stated above. The protein solutions were then dialyzed against 20 mM Hepes (pH 7.0) overnight, and fluorescence measurements were made on the dialyzed solutions. As expected, the λ_{max} values were shifted to those exhibited by GS–RNase T_1 and CM-RNase T_1 in absence of NaCl. The salt-induced conformational change (refolding) of the derivatives is therefore fully reversible.

Fluorescence Analysis of GS–RNase T_1 and CM-RNase T_1 in the Presence of Urea. GS–RNase T_1 and CM-RNase T_1 were incubated at 25 °C for 6 h at 0.54 μM (final concentration) in 20 mM Hepes (pH 7.0) containing 1 or 3 M urea in the absence or presence of 1.5 M NaCl. As for the analyses described above, the intrinsic fluorescence of the protein solutions was then measured after excitation at 278 or 295 nm, at 25 °C (Table 1). When incubated in the presence of 1 or 3 M urea in the absence of NaCl, GS–RNase T_1 and CM-RNase T_1 show no changes in their λ_{max} values or intrinsic fluorescence, even in the presence of the higher concentration of urea, thus confirming that even in absence of denaturant they are in a denatured state.

Table 1: Effect of Urea on λ_{max} Values of GS–RNase T_1 and CM-RNase T_1 in 20 mM Hepes (pH 7.0) Containing 1.5 M NaCl, after Excitation at 278 nm

	λ_{max}		
	0 M urea	1 M urea	3 M urea
GS–RNase T_1	322.8	338.1	348.6
CM-RNase T_1	324.4	346.5	349.7

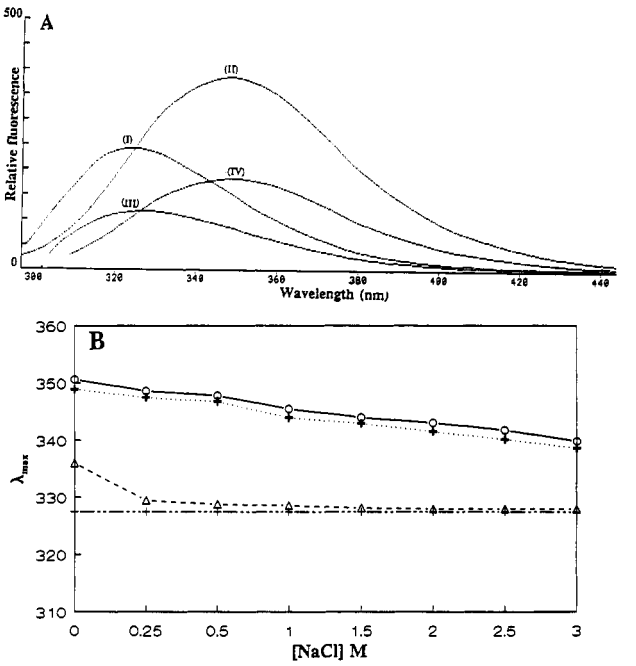

FIGURE 4: (A) Fluorescence emission spectra of native a-lactalbumin (I and II) and GS–α-lactalbumin (II and IV) in 20 mM Hepes (pH 7.0). Fluorescence emission was measured after excitation at 280 (I and II) or 295 nm (III and IV) at a protein concentration of 1.8 μM. (B) λ_{max} values of α-lactalbumin (standard conditions, 10 mM $CaCl_2$ (+); standard conditions, 1 mM EDTA (Δ)) and GS–α-lactalbumin (standard conditions, 10 mM $CaCl_2$ (+); standard conditions, 1 mM EDTA (O)) were plotted versus varying concentrations of NaCl. Fluorescence emission was measured after excitation at 295 nm.

As shown above, GS–RNase T_1 and CM-RNase T_1 regain the native λ_{max} in the presence of 1.5 M NaCl. When incubated in the presence of 1.5 M NaCl and 1 M urea, both of the modified proteins exhibited a shift toward the denatured λ_{max} value, together with a decrease in the intrinsic fluorescence compared to that in absence of urea. In the presence of 1.5 M NaCl and 3 M urea, GS–RNase T_1 and CM-RNase T_1, by fluorescence criteria, appeared to be fully unfolded. The data presented in Table 1 clearly show that the presence of urea counteracts the stabilizing effect of 1.5 M NaCl on GS–RNase T_1 and CM-RNase T_1.

Fluorescence Analysis of α-Lactalbumin and GS–α-Lactalbumin in the Presence of NaCl. Figure 4A shows the fluorescence spectra obtained for the native and modified lactalbumins under standard conditions (20 mM Hepes, pH

7.0), after excitation at 280 and 295 nm. When excited at 280 nm, native α-lactalbumin shows intrinsic fluorescence emission with λ_{max} at 323.6 nm, whereas GS–α-lactalbumin shows a greater emission intensity than the native form with a maximum at 349.7 nm, which is close to the λ_{max} of free tryptophan. When excited at 295 nm, native α-lactalbumin and GS–α-lactalbumin display a slight red shift in their λ_{max} values to 327.5 and 350.6 nm, respectively, together with a 2-fold decrease in their fluorescence intensities. As before, the two excitation wavelengths were used to distinguish the contributions of Tyr and Trp residues to the fluorescence emission, and the results confirmed that the intrinsic fluorescence of α-lactalbumin primarily reflectsthe environment of its four tryptophan residues (Trp26, Trp60, Trp104, and Trp118). The parameters determined, λ_{max} and relative fluorescence emission, demonstrate that the GS–α-lactalbumin is in a denatured state, as the fluorescence emission is known to be quenched in the native protein (Ewbank & Creighton, 1993b).

α-Lactalbumin and GS–α-lactalbumin were incubated at 25 °C for 6 h at a 1.8 μM final concentration in 20 mM Hepes (pH 7.0) containing various concentrations of NaCl in the absence or presence of 10 mM $CaCl_2$ or 1 mM EDTA. The intrinsic fluorescence emission spectra of the solutions were measured after excitation at 280 and 295 nm at 25 °C.

When incubated in the presence of increasing concentrations of NaCl, in 20 mM Hepes (pH 7.0) and 10 mM $CaCl_2$, the native protein showed no changes in intrinsic fluorescence or in the λ_{max} due to the presence of the salt, as shown in Figure 4B. By contrast, in the presence of 1 mM EDTA instead of 10 mM $CaCl_2$, conditions that have been reported to destabilize the native conformation (Ewbank & Creighton, 1993a,b), α-lactalbumin showed, as expected, a red shift in its λ_{max} (Figure 4B) and an increase in the fluorescence intensity compared to that in standard conditions, thus confirming that the dissociation of Ca^{2+} destabilizes the native conformation. In the presence of increasing concentrations of NaCl, the Ca^{2+}-free protein shows a [NaCl]-dependent blue shift in its λ_{max} (Figure 4B) and a decrease in the fluorescence intensity, but it does not completely regain the λ_{max} and the intensity shown in presence of excess Ca^{2+}. The native protein without the addition of either $CaCl_2$ or EDTA behaved indistinguishably from that in the presence of added $CaCl_2$ (data not shown), suggesting that the native protein retained firmly-bound Ca^{2+}.

Figure 4B also illustrates the behavior exhibited by the GS–α-lactalbumin incubated in 20 mM Hepes (pH 7.0) in the presence of varying concentrations of NaCl. The mixed disulfide, both in the presence of $CaCl_2$ and in the presence of EDTA, showed an emission maximum close to 350 nm, which is characteristic of a fully unfolded protein. Upon the addition of NaCl, the observed fluorescence maximum underwent a blue shift, and the fluorescence intensity showed a corresponding decrease; these changes are indicative of a shift toward the fluorescence characteristics of the native folded state, but this state is not reached, even in the presence of 3 M NaCl. The properties of GS–α-lactalbumin at all concentrations of NaCl are intermediate between those of the unfolded and folded conformations; the tryptophans of GS–α-lactalbumin are in fact still in a non-native but already more hydrophobic environment than in the fully unfolded state. As might be expected, at any given concentration of NaCl, the derivative in the presence of $CaCl_2$ appears marginally more folded than that in the presence of EDTA. Interestingly, however, the derivative, when incubated in absence of both $CaCl_2$ and EDTA, behaved indistinguishably from a preparation in the presence of EDTA (data not shown), implying that the derivative has a low affinity for Ca^{2+} and does not retain bound Ca^{2+} through the preparative procedure.

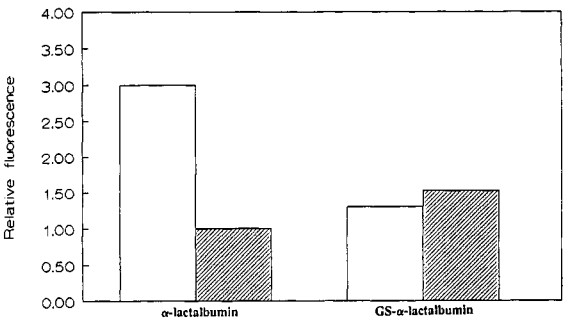

FIGURE 5: Binding of ANS by α-lactalbumin and GS–α-lactalbumin in the presence of 1 mM EDTA (open bars) or 10 mM $CaCl_2$ (hatched bars).

Table 2: Activity Data

protein	activity (%) 20 mM Hepes (pH 7.0)[a]	20 mM Hepes (pH 7.0) and 1.5 M NaCl[b]
RNase T_1	100	100
GS–RNase T_1	0	2.5
CM-RNase T_1	0	1.6

[a] RNA solution in 50 mM Tris-HCl, 25 mM KCl, and 5 mM $MgCl_2$ (pH 7.5). [b] RNA solution in 50 mM Tris-HCl, 25 mM KCl, 5 mM $MgCl_2$, and 1.5 M NaCl (pH 7.5).

ANS Binding Analysis. ANS was titrated with native α-lactalbumin and GS–α-lactalbumin in the presence of 10 mM $CaCl_2$ or 1 mM EDTA. A linear correlation between protein concentration and ANS fluorescence was obtained in all cases. As shown in Figure 5, in the presence of 10 mM $CaCl_2$, native α-lactalbumin had the expected low affinity for ANS (Ewbank & Creighton, 1993b). In the presence of 1 mM EDTA, native α-lactalbumin showed a significant increase in ANS fluorescence. ANS has been used to probe the existence of molten globule states, as the enhancement of ANS fluorescence by this state has been reported to be between 2- and 10-fold more than the low level seen with the native and fully unfolded states (Ptitsyn et al., 1990; Semisotnov et al., 1991). The effect of EDTA indicates that the dissociation of Ca^{2+} induces the formation of a molten globule state in the protein, as previously noted (Ewbank & Creighton, 1993a,b).

Figure 5 also illustrates the binding with ANS exhibited by GS–α-lactalbumin. The modified protein showed a low affinity for ANS in the presence of either 10 mM $CaCl_2$ or 1 mM EDTA. In combination with the results (above) on intrinsic fluorescence properties, this result indicates that the GS derivative is in a fully unfolded state both in the presence and absence of the stabilizing cation ligand.

ANS was also titrated with native RNase T_1 and GS–RNase T_1 under the standard conditions (20 mM Hepes, pH 7.0, 25 °C). The native and modified proteins displayed a very low affinity for ANS (data not shown), confirming that they are in a fully folded state and a fully unfolded state, respectively, as suggested by the intrinsic fluorescence data (above).

Activity Data on GS-RNase T_1 and CM-RNase T_1. Activity measurements were made for native RNase T_1, GS–RNase T_1, and CM-RNase T_1 at 30 °C after incubation of the samples in 20 mM Hepes (pH 7.0) at 25 °C; the results are presented in Table 2. It can be seen that breaking the disulfide bonds and modifying the cysteine residues lead to loss of enzymatic activity under our experimental conditions.

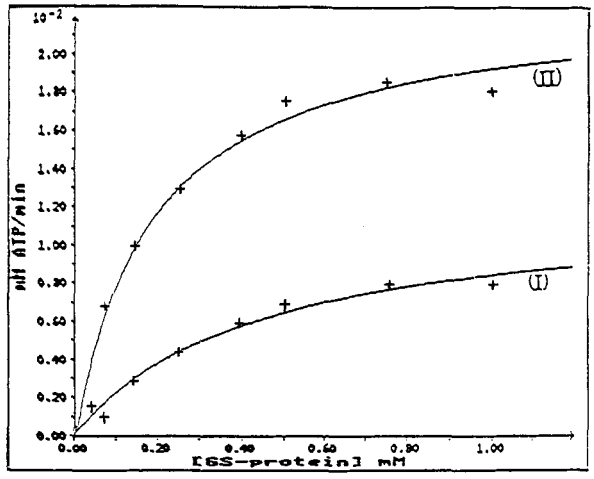

FIGURE 6: BiP binding to GS–RNase T_1 (I) and GS–α-lactalbumin (II). Purified BiP (4 mg) was incubated with GS–RNase T_1 or GS–α-lactalbumin (0–1 mM) in 20 mM Hepes (pH 7.0). BiP ATPase activity was determined by a coupled assay, as described in the Materials and Methods section.

The activities were also measured under the same conditions but in the presence of 1.5 M NaCl, after incubation of the samples in 20 mM Hepes and 1.5 M NaCl (pH 7.0) (Table 2). Here, the activities of GS–RNase T_1 and CM-RNase T_1 were 2.5% and 1.6%, respectively, that of the unmodified protein, showing that refolding in the presence of 1.5 M NaCl, as illustrated by the fluorescence analysis, is accompanied by a limited regain of the enzymatic activity.

Stimulation of BiP ATPase Activity in the Presence of GS-RNase T_1 and GS-α-Lactalbumin. The rate of ATP hydrolysis by BiP was measured in the presence of increasing concentrations of GS–RNase T_1 and GS–α-lactalbumin. Figure 6 shows that ATP is hydrolyzed in the presence of both GS–RNase T_1 and GS–α-lactalbumin and that the rate is dependent on the concentration of the derivatized proteins. In the absence of the effectors, a low rate of ATP hydrolysis was measured (3.4 μM ATP/min); this basal BiP activity was subtracted when calculating the ATP hydrolysis due to binding of unfolded proteins. The kinetic data gave a linear double-reciprocal plot for both of the proteins, yielding a K_m of 0.355 mM for GS–RNase T_1 and a K_m of 0.163 mM for GS–α-lactalbumin. The same analysis was also performed on the native proteins as a reference. Neither RNase T_1 nor α-lactalbumin was able to elicit BiP ATPase activity, thus confirming that members of the hsp70 family interact with a variety of unfolded protein targets, but not with their properly folded counterparts (Palleros et al., 1991).

DISCUSSION

The majority of secretory and extracellular proteins contain disulfide bonds. In the generation of the native states of these proteins, folding and protein disulfide formation are intimately interrelated processes (Creighton et al., 1993). Fully reduced derivatives are the natural starting materials for studies on the folding of such proteins, but these derivatives tend toward ill-controlled oxidation and, in many cases, they are poorly soluble. The coupling of folding and native disulfide formation can also be studied with "scrambled" protein substrates that are incompletely folded and contain non-native disulfide bonds, but these are difficult to prepare reproducibly and are generally heterogeneous and incompletely defined in chemical terms (Hillson et al., 1984). In some biotechnological applications, the Cys residues of recombinant proteins are converted to *S*-sulfonates prior to refolding to optimize the formation of the native disulfide isomer (Marston, 1986; Fischer et al., 1993). Mixed disulfides between proteins and low molecular weight thiols provide an interesting alternative; it should be possible to prepare defined derivatives quantitatively, and they are stable, provided that no thiols are present to promote thiol/disulfide interchange. Furthermore, provided that the low molecular weight thiol is itself polar, the derivatives should be comparatively water-soluble. In this paper, we have prepared mixed disulfides between model proteins and the physiological thiol, glutathione, characterized them in terms of chemical structure and conformational properties in solution, and shown that they are good models for unfolded proteins and so can be used to study the coupled processes of folding and protein disulfide formation.

The conversion of all of the free thiols of a reduced protein to mixed disulfides requires the addition of a great excess of disulfide oxidant. After formation of the first mixed disulfide on a protein, the intramolecular attack of a free protein thiol on this disulfide, to eliminate the low molecular weight thiol and form a protein disulfide, is greatly favored. A great excess of the low molecular weight disulfide reagent is necessary to suppress this competing process, leading to the formation of protein disulfide bonds. The data presented here clearly show that RNase T_1 and α-lactalbumin can be converted to stable mixed disulfide derivatives in which each cysteine residue had reacted with the exogenous glutathione.

The ES/MS analysis and DTNB titration data showed that at least 90% of the GS mixed disulfide products isolated after the reaction of a 1000-fold excess of GSSG with both RNase T_1 and α-lactalbumin were the required GS derivatives of the reduced protein, with no intramolecular protein disulfide bonds. In preliminary experiments with other model proteins, rather higher proportions of the undesired derivatives with intramolecular protein disulfide bonds were recovered; in this paper, we have concentrated on analyzing the properties of the glutathione derivatives of two model proteins only. The preparation of the mixed disulfides was carried out with a final yield of 60% (mg of derivative/mg of starting native protein). Due to the high purity of the two derivatives, they were used without further purification.

The modified proteins were found in an appreciably denatured state, under native conditions, by the observed fluorescence parameters: λ_{max} and relative fluorescence emission (Pace, 1986; Ewbank & Creighton, 1993b). The low affinity shown for the hydrophobic fluorescent dye ANS was also indicative that GS–RNase T_1 and GS–α-lactalbumin were in an unfolded state. Enzyme activity data indicated, in addition, that breaking the S–S bonds and derivatizing the cysteines of RNase T_1 led to the complete loss of the enzymatic activity of the protein. The conformational and stability properties of the mixed disulfides were then analyzed by intrinsic fluorescence in the presence of varying concentrations of NaCl.

Salt can in fact influence the stability of proteins in a variety of ways (von Hippel & Schleich, 1969) with a wide range of interactions, depending on the kind of salt and the solvent conditions (Arakawa & Timasheff, 1984). RNase T_1 has been shown to be markedly stabilized by NaCl (Oobatake et al., 1979), mainly because of the relatively weak binding of cations and anions to sites on its surface. RNase T_1 is in fact a very acidic protein, and the binding of cations seems to help compensate for the shortage of basic residues (Pace & Grimsley, 1988).

Fluorescence studies presented in this paper confirmed the results of the published work on CM-RNase T_1 (Pace et al.,

1988), showing that it can indeed refold to a nativelike structure in the presence of high concentrations of NaCl. More surprisingly, even GS–RNase T_1 has been shown to regain the native λ_{max} value at high concentrations of NaCl and to exhibit an increase in the intrinsic fluorescence intensity as the NaCl concentration increases, without completely recovering the intrinsic fluorescence of the native form, which is probably due to the effect of the exogenous groups present in the molecule.

The single tryptophan (Trp59) in RNase T_1 is completely buried near the center of the molecule, and several of the nine tyrosine residues transfer excitation energy to it (Pongs, 1970). In light of this, it is very interesting that the maximum emission wavelength of the intrinsic fluorescence of the native protein and the GS–RNase T_1 do not differ significantly in the presence of high concentrations of NaCl. This indicates that, in the presence of NaCl, the Trp59 of GS–RNase T_1 is sequestered in a hydrophobic environment. On the other hand, the attached glutathione moieties must have some effect on the time-averaged conformation or on the dynamics of the protein, since the fluorescence intensity of the derivative under these conditions is not equal to that of the native protein, and the derivative shows only a small fraction of the native enzyme's catalytic activity. The 6–103 disulfide bond is indeed completely buried (Pace et al., 1988), and it is possible that the increase in volume due to the glutathione moieties is not tolerated in the tightly-packed interior of the protein, disrupting the enzyme activity without significantly influencing the immediate environment of Trp59.

It is worth underlining that the removal of the salt from the solution by dialysis causes the modified proteins to readopt the denatured state, thus confirming that the regain of native tertiary structural properties is entirely due to the presence of the salt. The presence of a denaturating agent such as urea can, in addition, counteract the stabilizing effect of NaCl on the modified proteins.

In contrast to GS–RNase T_1, GS–α-lactalbumin was unable to fold to a nativelike structure in the presence of NaCl, but it seems to adopt an average conformation that is intermediate between the fully native and fully denatured states. Since this protein contains four Trp residues, it is not easy to interpret the steady-state fluorescence emission properties in structural terms.

α-Lactalbumin is dramatically stabilized by binding Ca^{2+} ions (Hiraoka et al., 1980); the bound Ca^{2+} interacts with seven oxygen ligands: three carboxylates from Asp residues, two backbone carbonyls, and two water molecules (Acharya et al., 1989). The effects of altering the free Ca^{2+} ion concentration, by the addition of $CaCl_2$ or EDTA, on the fluorescence properties of native α-lactalbumin and the GS derivative indicated that while Ca^{2+} stabilizes the folded conformation of the unmodified protein, it does not significantly promote the folding of the GS derivative, suggesting that the derivative has a low affinity for Ca^{2+}. Our data on unmodified α-lactalbumin confirm the results of Ewbank and Creighton (1993b). The data on the effects of Ca^{2+} on the fluorescence of the GS derivative are consistent with previous conclusions of Ikeguchi et al. (1986). They showed that, up to 1 mM Ca^{2+}, the binding of Ca^{2+} occurs to the molecules in the native state, with no interaction detected between Ca^{2+} and unfolded α-lactalbumin, but at concentrations of Ca^{2+} higher than 1 mM, such as the concentration used in our experimental conditions, the binding of Ca^{2+} occurs even to the molecules in the unfolded state.

In both the absence and presence of Ca^{2+}, high concentrations of NaCl had a moderate influence on the conformation of GS–α-lactalbumin, causing a small blue shift in the λ_{max} value and a decrease in the fluorescence intensity, but the protein did not regain the native conditions. A reasonable explanation for this result is the fact that the Ca^{2+} sites of α-lactalbumin can also bind Na^+ ions, but with a lower affinity: $K_b = 2.9 \times 10^9$ M^{-1} for Ca^{2+} ions and 1240 M^{-1} for Na^+ ions (Segawa & Sugai, 1983; Mitani et al., 1986; Pace, 1990).

Finally, GS–RNase T_1 and GS–α-lactalbumin were shown to be capable of stimulating BiP ATPase activity. The kinetic data yield a K_m of 0.355 mM for GS–RNase T_1 and a K_m of 0.163 mM for GS–α-lactalbumin. To the best of our knowledge, these are the first K_m data reported for unfolded proteins interacting with BiP. Both of the calculated values are in the 0.01–1 mM range reported for several synthetic peptides binding to BiP (Flynn et al., 1989). GS–α-lactalbumin appears to show a stronger interaction with BiP than does GS–RNase T_1. It is not clear whether this derives from overall differences in the properties of the two proteins (charge, extended nature of the unfolded state) or a specific difference in the positions of the BiP binding sequences. The content of aliphatic amino acids, such as Leu, Ile, and Met, in the α-lactalbumin sequence is higher than that of RNase T_1. The high affinity of BiP for GS–α-lactalbumin could then be due to the fact that the BiP peptide binding site has a preference for the above-cited aliphatic side chains (Flynn et al., 1991). On the other hand, both proteins contain two heptapeptide sequences that are strong candidates for BiP binding (M.-J. Gething, personal communication), according to the consensus binding criteria identified by Blond-Elguindi et al. (1993).

Work is in progress to study the full refolding of the mixed disulfides by introducing thiol compounds to permit thiol/disulfide interchange and analyzing the effect of PDI and/or BiP on the rate and/or yield of refolding.

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